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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>G01N 33/483, 35/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/63342</b> <b>(43) International Publication Date:</b> 9 December 1999 (09.12.99)
<b>(21) International Application Number:</b> PCT/US99/12263 <b>(22) International Filing Date:</b> 2 June 1999 (02.06.99)  <b>(30) Priority Data:</b> 60/087,673 2 June 1998 (02.06.98) US  <b>(71) Applicant (for all designated States except US):</b> VENTANA MEDICAL SYSTEMS, INC. [US/US]; 3865 North Business Center Drive, Tucson, AZ 85705 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MEHTA, Parula [US/US]; 7374 N. Mona Lisa Road #10103, Tucson, AZ 85741 (US). GRAHAM, Marshal [US/US]; 4858 East Winsett Street, Tucson, AZ 85711 (US). POMERANTZ, Anlouise [US/US]; 4656 El Camino de Cerro, Tucson, AZ 85705 (US).  <b>(74) Agent:</b> HARPER, David, S.; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US).		<b>(81) Designated States:</b> JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD FOR STAINING BIOLOGICAL SPECIMENS BY COMBINING UNSTABLE REAGENTS ON A MICROSCOPE SLIDE  <b>(57) Abstract</b>  The present invention relates to automated methods for staining biological materials on a slide comprising the use of component histochemical solutions mixed directly on a biological sample of interest. The method comprises providing at least two stable solutions that together comprise an unstable staining solution, sequentially delivering the stable solutions to a biological sample of interest on a surface, and mixing the stable solutions directly on the biological material of interest to effectuate staining of the material.		

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**Method for Staining Biological Specimens by Combining Unstable Reagents on  
a Microscope Slide**

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**Related Applications**

This application is a continuation in part of U.S. Application Serial No.  
60/087,673 filed June 2, 1998.

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**Field of the Invention**

The present invention relates to formulations for component histochemical  
staining solutions as well as methods for formulating, storing, and combining  
15 components of unstable histochemical staining solutions. In particular, the present  
invention relates to methods for combining component histochemical solutions  
directly on the biological sample of interest.

**Background**

20

Histochemistry is the science by which chemical reactions are used to  
identify particular substances within a cell. One method of identifying particular  
substances in the cell is by staining the cells with chemicals (dyes) that make such  
substances or structures more visible. Perhaps the most common stain materials are  
hematoxylin and eosin. Hematoxylin is utilized to stain the nuclei of the cell dark  
25 blue while eosin stains the cell cytoplasm various shades of red or yellow that  
contrast with the blue stain of the nuclei. Other stains can be used to identify other  
substances within the cell such as collagen, elastin, mucin, ferric iron, and other  
substances. Still other stains can be used to identify agents that infect the human

body such as bacteria and fungi. Many of the stains used to identify certain substances and/or structures within or outside the cell require the used of stains that are unstable, toxic, and generally messy and difficult to work with.

Presently, many automated and manual histochemical staining protocols  
5 require the pre-mixing of two or more solutions prior to staining sample tissue. In many cases the mixing of several solutions to prepare a single solution for staining produces a staining solution that is inherently unstable. Instability may manifest itself by the appearance of precipitates or films in the staining solution. For example, many silver staining solution are photolabile. Ammoniacal silver solutions  
10 degrade rapidly and a silver residue can be observed on top of the solution within hours of mixing. The formation of films and precipitates negatively affects the staining of the tissue and therefore decrease the accuracy of histochemical testing. Furthermore, the daily preparation of fresh histochemical staining solutions is time consuming. It may also be costly since expensive reagents such as silver nitrate may  
15 be squandered if staining solution is prepared and not used by the end of the day. Therefore, there exists a need for improved histochemical staining methods that employ unstable staining solutions.

The present invention obviates the need to prepare new staining preparations on a daily basis. The present invention permits the mixing of component  
20 histochemical staining solutions on a sample tissue slide, solutions that have previously been combined in the laboratory prior to staining a slide sample. Unlike the combined solutions which are unstable, the separated component solutions are stable for long periods. The component histochemical solutions of the present invention may be stored as separate solutions for long duration and may then be

combined on sample tissue that has been placed on a microscope slide. The results of tissue assays using the component histochemical staining solutions of the present invention are equal to or better than manual or automated methods utilizing completely mixed standard histochemical staining solutions.

5

### **Summary of the Invention**

The present invention relates to automated methods for staining biological materials on a slide comprising the use of component histochemical solutions combined directly on a biological sample of interest. In one embodiment, the method comprises providing at least two stable solutions that together comprise an unstable staining solution, sequentially delivering the stable solutions to a biological sample of interest on a planar surface, and combining the stable solutions directly on the biological material of interest to effectuate staining of the material. In specific embodiments, methods are provided for automated silver staining, iron staining, trichrome staining, and mucicarmine staining.

In various preferred embodiments, the biological material is selected from the group consisting of tissue sections, tissue culture cells, nucleic acids, proteins, and chromosomes; the unstable staining solution is selected from the group consisting of fungi staining solutions, silver staining solutions, trichrome staining solutions, mucin stains, mucicarmine staining solutions, iron staining solutions, Verhoff's staining solution, and Steiner staining solution, the solutions are mixed, and the mixing is accomplished by applying a gas stream to the at least first and second stable solutions on the biological material.

### Detailed Description of the Preferred Embodiments

5           The present invention relates to automated methods for staining biological materials on a surface comprising the use of component histochemical solutions mixed directly on a biological sample of interest. There are many histochemical staining procedures that require the use of a staining solution that is made of several component solutions. These component solutions are mixed together prior to being  
10 placed on a microscope slide containing a tissue section of interest. In the present invention these component solutions are kept in separate containers and only mixed after placement of each solution successively on the biological material of interest. In a preferred embodiment, the solutions are mixed on the slide by an automated histochemical instrument and the concentrations of the solutions optimized for the  
15 instrument and the method of mixing. The methods of the present invention do not require mixing of the solutions, but such mixing speeds up and limits variation in the resulting solution.

As used herein, the term "solution" encompasses solutions, emulsions, and suspensions.

20           As used herein, the term "stable" means that the solution can be stored and re-used, and thus does not need to be made fresh prior to use. Preferably, a "stable solution" has a shelf-life of at least one week.

As used herein, the term "unstable" means that the solution exhibits diminished capacity to stain the target organism or tissue, upon standing for any  
25 period of time, even as little as one hour. For example, many silver staining

solutions are photolabile and heat labile. Similarly, many staining solutions change color or form precipitates or films as a result of oxidation, such as iron hematoxylin, and must be discarded after use. The methods of the invention apply to any unstable multi-component staining solution that can be made by mixing two or more stable sub-components together. Special stains color, or coat with metals, certain specific kinds of cells or cellular structures. This is done by applying, in sequence, dyes and other chemicals (oxidizers, reducing agents, metals) until the targeted staining is accomplished. Some stains employ as many as 10 different solutions. Each solution is termed a component of the stain.

10        Some individual components of the stain are made of "sub-components". If a final formulation of a solution cannot be stored until it is needed for use, then the separate ingredients must be made into "stock solutions" and combined immediately before use. The combined solution is not "stable", so it must be used within a short time, before it degrades and does not perform its function in the staining procedure.

15        This "unstable" combined solution is called a "working solution". A single component of a stain may have multiple sub-components that can be combined in a variety of ways to achieve the desired result.

          The method of the present invention can be used with any histochemical solution that exhibits diminished capacity to stain the target organism or tissue, upon standing for any period of time, even as little as one hour. Such unstable multi-component staining solutions include, but are not limited to fungi staining solutions, silver staining solutions, iron staining solutions, iron hematoxylin solutions, trichrome staining solutions, mucin stains, mucicarmine staining solutions, Verhoff's staining solution, amyloid staining solutions, and Steiner staining solution.



See for example, Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (McGraw-Hill, New York, Lee G. Luna, Ed. (1968)) (AFIP Manual); Theory and Practice of Histological Techniques (Churchill Livingstone, NY, ed. by Bancroft and Stevens, 4th edition, 1996); both incorporated by reference  
5 herein in their entirety. The method of the present invention can be used for bi-component, tri-component (as in the example above) and other multi-component unstable histochemical solutions.

For example, the conventional Grocott's method for fungi (GMS) requires a number of solutions to accomplish the staining of fungal tissue (chromic acid,  
10 sodium bisulfite, gold chloride, sodium thiosulfate, light green solution). In addition, this staining protocol requires the use of a methanamine-silver nitrate-borax solution which is made by mixing a silver nitrate-methanamine stock solution (0.25% silver nitrate, 2.85% methanamine) with a 5% borax solution to produce a working methanamine-silver nitrate solution (0.125% silver nitrate, 1.425%  
15 methanamine, 0.2% borax). While the stock methanamine-silver nitrate solution is stable, the working solution is unstable and thus must be made fresh every day.

In the present invention, the silver nitrate solution is kept separate from the methenamine-borax solution until the two solutions are mixed directly on the tissue of interest. In a preferred embodiment of the present invention the silver nitrate  
20 solution is comprised of from about 0.2% to about 1.0% silver nitrate. In a preferred embodiment of the present invention the methenamine-borax solution is from about 2.0% to about 4.0% methenamine and from about 0.2% to about 0.6% borax in distilled water. In a preferred embodiment of the present invention the silver nitrate solution is added to the sample and after addition of a liquid coverslip solution, an

equal volume of methenamine-borax solution is added to the sample. Also, each of the three sub-components can be added as separate solutions to the sample. Furthermore, one of skill in the art will recognize that a methenamine-silver nitrate stock can be mixed on the tissue with the borax solution.

5           Similarly, conventional ammoniacal silver staining requires the use of both silver nitrate and ammonium hydroxide/sodium hydroxide solutions. While the stock silver nitrate and ammonium hydroxide/sodium hydroxide solutions are stable, the combined working solution is unstable and thus must be made fresh every day.

          In the present invention, the silver nitrate solution is kept separate from the  
10   ammonium hydroxide/sodium hydroxide solution until the two solutions are mixed directly on the tissue of interest. In a preferred embodiment of the present invention the silver nitrate solution is comprised of from about 0.2 % to about 1.0 % silver nitrate. In a preferred embodiment of the present invention the ammonium hydroxide/sodium hydroxide solution is from about 0.3 % to about 1 % ammonium  
15   hydroxide and from about 0.1% to about 0.5% sodium hydroxide in distilled water. Also, each of the three sub-components can be added as separate solutions to the sample.

          Trichrome staining and mucicarmine staining require both Weigerts iron hematoxylin A and B solutions. While the stock Weigerts A and B solutions are  
20   stable, the combined working solution is unstable and thus must be made fresh every day.

          In the present invention, the Weigerts A solution is kept separate from the Weigerts B solution until the two solutions are mixed directly on the tissue of interest. In a preferred embodiment of the present invention the Weigerts A solution

is comprised of from about 0.7 % to about 1.5 % hematoxylin in 95% alcohol. In a preferred embodiment of the present invention the Weigerts B solution is from about 0.7% to about 1.5 % aqueous ferric chloride and from about 0.5 % to 1.5 % HCl in distilled water. In a preferred embodiment of the present invention the Weigerts B solution is added to the sample and after addition of a liquid coverslip solution, an equal volume of Weigerts A solution is added to the sample.

Gomori's iron staining require both potassium ferrocyanate and hydrochloric acid solutions. While the stock solutions are stable, the combined working solution is unstable and thus must be made fresh every day.

In the present invention, the potassium ferrocyanate is kept separate from the hydrochloric acid solution until the two solutions are mixed directly on the tissue of interest. In a preferred embodiment of the present invention the potassium ferrocyanate solution is comprised of from about 8 % to about 12 % potassium ferrocyanate in 95% distilled water. In a preferred embodiment of the present invention the hydrochloric acid solution is from about 15 % to about 30 % HCl in distilled water.

In the methods of the invention, the solutions can be contacted with the biological material for widely varying periods of timing to accomplish the object of staining the specimen. In one embodiment, the solution is contacted with the biological specimen for between about one second and about one hour, preferably for between about 10 seconds and 45 minutes, and most preferably for between about one minute and 30 minutes.

The methods of the present invention can be performed over a wide temperature range. In one embodiment, the methods can be performed at between

about 20°C to about 90°C; more preferably at between about 40°C to about 70°C;  
and most preferably between about 50°C and about 60°C.

The parameters of temperature at which the staining is carried out, and the duration of contacting the biological specimen with the solution, can be varied  
5 extensively depending upon the stain, the biological specimen, and the instrumentation used, as will be appreciated by one of skill in the art.

In a preferred embodiment of the present invention the solutions are added to the sample tissue by an automated mechanism which can mix the solutions on the slide. Such automated instruments include those described in U.S. Patent Nos.  
10 5,595,707; 5,654,199; 5,654,200 and 5,650,327 herein incorporated by reference in their entirety. The particular concentrations of reagents in the component solutions can be optimized by standard experimental design to provide optimum ranges of concentrations, oxidation/reduction potentials, ionization, and/or pH.

In a preferred embodiment, the methods of the present invention are  
15 automated. Manual and most robotic staining is performed by dipping the slides into open vessels that are filled with pre-mixed solutions of dyes and chemicals. A variant of this technique is flooding chambers containing the slides with the pre-mixed solutions. In contrast, in the method of the present invention, the slide or other surface is itself used as the container for the staining solution. The slides are  
20 positioned flat, biological material side up, and aliquots of staining solutions are sequentially delivered and mixed on the biological material. Instrumentation for conducting such automated staining includes, but is not limited to the NexEs™ system (Ventana Medical Systems, Tuscon, AZ) and that disclosed in U.S. Patent

Nos. 5,654,200, 5,650,327, 5,654,199, and 5,595,707, all hereby incorporated by reference in their entirety.

In a preferred embodiment, methods are used to apply a layer over the "pool" of staining solution to prevent evaporation, regulate temperature, and enhance mixing, such as that described in U.S. Patent Nos. 5,654,200, 5,650,327, 5,654,199, and 5,595,707, all hereby incorporated by reference in their entirety. In a particularly preferred embodiment, the layering method comprises (a) covering the sample with an aqueous surface layer by applying an aqueous solution to a planar surface adjacent a biological sample mounted thereon; and (b) covering the aqueous surface layer with an evaporation inhibiting liquid layer by applying the evaporation inhibiting liquid to the planar support surface adjacent the biological sample in an amount sufficient to form a continuous layer of evaporation inhibiting liquid over the sample. The evaporation inhibiting liquid is substantially water-insoluble, substantially water-immiscible and substantially non-viscous; has a specific gravity less than water, and a boiling point above 50o C.; and is devoid of chemical characteristics which would significantly interfere with biochemical reactions carried out on the sample. The biological sample can then be optionally treated (c) with an aqueous reagent solution by applying the reagent solution to the planar support surface adjacent the biological sample. The reagent solution flows to the biological sample under the evaporation inhibiting liquid layer, and the sample is continuously protected from dehydration by the evaporation inhibiting layer.

The methods of the present invention include mixing the stable solutions on the surface of the biological sample. In a preferred embodiment, this is accomplished by applying at least one gas stream to an area of the surface of the

evaporation inhibiting liquid layer between the center of the evaporation inhibiting layer and the edge of the planar support surface, the gas stream having a central axis forming an acute angle with the planar support surface. According to one embodiment of the present invention, the reagent solution is preferably stirred by a vortex formed by applying two off-center gas streams, flowing in opposite directions, to the surface of the evaporation inhibiting liquid layer. According to a further embodiment of the present invention, the stable solutions are stirred by a vortex formed by applying a single gas stream along a longitudinal edge of the slide, the gas stream originating from the distal edge of the slide.

Biological materials that can be stained by the methods of the invention include, but are not limited to tissue sections, tissue culture cells, cell components, including cell organelles, chromosomes, nucleic acids, carbohydrates, lipids, and proteins, smears of blood, sputum, and other body fluids, excretions and secretions, and micro-organisms including parasites, viruses, bacteria, and fungi.

The methods of the present invention can also utilize newly developed stains. A generic method for applying the subject invention to most any stain comprises:

1. Reviewing the staining literature and selecting a particular staining protocol.
2. Evaluate the instrument platform to be used to determine parameters and limitations in time, temperature and rinsing and mixing events available on the instrument.
3. Adapt the staining procedure to conform to the instrument parameters. Example: If more or less time is needed, but not available, then increase or decrease, temperature or reagent concentration.
4. Test the modified staining procedure and evaluate the result.
5. If the result is sub-optimal, then identify the component responsible for the problem.
6. Substitute or reformulate the reagent to compensate for the problem.
7. Retest and reevaluate in a loop until stain is optimized.

This procedure will permit one of skill in the art to apply the subject invention to most any staining procedure, including those described in Theory and Practice of Histotechnology, Dezna C Sheehan H.T (ASCP), Battelle Press, 2nd ed., 1980; and Laboratory Histopathology, Anthony E. Woods & Roy C. Ellis, Churchill Livingstone, 1st ed., 1994; both incorporated by reference herein in their entirety.

The following Examples are presented for illustrative purposes only and are not intended, nor should they be construed as limiting the invention in any way. Those skilled in the art will recognize that variations on the following can be made without exceeding the spirit or scope of the invention.

10

**Example 1**  
*Grocott's Method for Fungi (GMS) Staining*

A solution of 0.35% silver nitrate was made by adding 0.35g silver nitrate to 100 ml de-ionized water. A solution of 3.0% methenamine and 0.4% borax was made by dissolving 3g methenamine and 0.4g of borax in 100 ml of distilled water. A solution containing 0.5% sodium bisulfite was made by dissolving 0.5g of sodium bisulfite in 100 ml of distilled water. A solution containing 3.75% chromic acid was made by dissolving 3.75g of chromium trioxide in 100 ml of distilled water. A solution containing 0.2% gold chloride was made by dissolving 0.2g of gold chloride in 100 ml of distilled water. A solution containing 2.0% sodium thiosulfate was made by dissolving 2.0g of sodium thiosulfate in 100 ml of distilled water. A 0.05% light green solution was made by diluting 25 ml of stock solution (2g of light green dissolved in 99 ml distilled water and 1 ml glacial acetic acid) in 100 ml of de-ionized water. 200 µl of the silver nitrate solution and 200 µl of the methenamine-borax solution were dispensed onto a tissue sample mounted on a microscope slide

using an automated histochemical dispensing apparatus (Ventana Medical Systems, Inc., Tucson Arizona). The sample tissue, *Aspergillus cyptococcus* was prepared for staining by standard technique. The sample tissue was place in the automated histochemical staining instrument and the following protocol was used in staining  
 5 the tissue.

In this example, as well as the four succeeding examples, the wash solution was comprised of 0.2% Tween 20 in de-ionized water (pH to  $7.0 \pm 0.5$ ). Liquid coverslip is 99.99% Norpar 15 and less than 0.01% oil red O.

10

**TABLE 1**

- |    |     |  |
|----|-----|--|
|    | 1.  | Warm-up rinse buffer to 41.0° C  |
|    | 2.  | Rinse slide  |
|    | 3.  | Adjust slide volume  |
| 15 | 4.  | Apply liquid coverslip   |
|    | 5.  | Warm slide chamber to 60.0° C  |
|    | 6.  | Rinse slide  |
|    | 7.  | Adjust slide volume  |
|    | 8.  | Apply liquid coverslip   |
| 20 | 9.  | Start timed steps  |
|    | 10. | Rinse slide  |
|    | 11. | Adjust slide volume  |
|    | 12. | Apply 200µl of 4% chromic acid and incubate for 15 minutes                     |
|    | 13. | Apply liquid coverslip   |
| 25 | 14. | Rinse slide  |
|    | 15. | Adjust slide volume  |
|    | 16. | Apply 200µl of 0.5% sodium bisulfite and incubate for 3 minutes                |
|    | 17. | Apply liquid coverslip   |
|    | 18. | Rinse slide  |
| 30 | 19. | Rinse slide  |
|    | 20. | Adjust slide volume  |
|    | 21. | Apply 200 µl of 0.5% silver nitrate solution and incubate for 3 minutes        |
|    | 22. | Apply liquid coverslip   |
| 35 | 23. | Apply 200 µl of 4% methanamine/0.4% borax solution and incubate for 18 minutes |
|    | 24. | Apply liquid coverslip   |
|    | 25. | Rinse slide  |
|    | 26. | Adjust slide volume  |



27. Apply liquid coverslip
28. Apply 200µl of 0.2% gold chloride and incubate for 3 minutes
29. Apply liquid coverslip
30. Rinse slide
- 5 31. Adjust slide volume
32. Apply 200µl of 2.5% sodium thiosulfate solution and incubate for 3 minutes
33. Apply liquid coverslip
34. Rinse slide
- 10 35. Adjust slide volume
36. Apply liquid coverslip
37. Apply 200µl of light green solution and incubate for 3 minutes
38. Apply liquid coverslip
39. Rinse slide

15

A visual comparison between the tissue prepared using the automated protocol as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for GMS described in the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (McGraw-Hill, New York, Lee G. Luna, Ed. (1968)) (AFIP Manual). Such comparison showed that the tissue stained with the component solutions on the automated system were cleaner, *i.e.* no black ring around the sample tissue. All tissue samples remained attached to the slide, whereas those stained manually started to lift off the slide. The staining contrast was better on the slides stained with the component solutions.

25

The silver nitrate solution and methanamine/borax solutions were stored at 4° C for three months, after which time the protocol in table 1 was re-run on similar tissue. A visual comparison was made between the tissue prepared as described above using the stored solutions and the manual staining of identical tissue with freshly made solutions. The staining comparison demonstrated that the tissue

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stained by the stored solutions run on the automated system were comparable or better than tissue stained manually with freshly made solutions.

5

**Example 2**  
*Ammoniacal Silver Staining*

A 10% silver nitrate stock solution was made by dissolving 10g of silver nitrate in de-ionized water. A working solution of 0.2% silver nitrate was made by diluting 2 ml of 10% stock solution with 48 ml of de-ionized water. An ammonium hydroxide/sodium hydroxide solution was made by dissolving 9.20 ml of 1N ammonium hydroxide and 3.60 ml of a 3% sodium hydroxide in 37.2 ml of de-ionized water. A solution containing 0.5% potassium permanganate was made by dissolving 0.5g of potassium permanganate in 100ml of distilled water. A solution containing 0.5% oxalic acid was made by dissolving 0.5g of oxalic acid in 100ml of distilled water. A solution containing 2.5% ferric ammonium sulfate was made by dissolving 2.5g of ferric ammonium sulfate in 100ml of distilled water. A solution containing 10% formalin was made by diluting 10 ml of concentrated formaldehyde (37-40%) in 90 ml distilled water . A solution of 0.2% gold chloride was made by dissolving 0.2g of gold chloride in 100ml of distilled water. A solution containing 2.0% sodium thiosulfate was made by dissolving 2g of sodium thiosulfate in 100 ml of distilled water. A solution of 1.5g/L nuclear fast red was made by dissolving 0.15g of nuclear fast red in 5% solution of aluminum sulfate (5g of aluminum sulfate in 100 ml distilled water) over heat. 200 $\mu$ l of 0.2% silver nitrate and 200 $\mu$ l of the ammonium hydroxide/sodium hydroxide solution were dispensed onto a tissue sample mounted on a microscope slide using an automated histochemical dispensing

apparatus (Ventana Medical Systems, Inc., Tucson Arizona). Sample liver tissue was prepared according to standard protocol. The sample tissue was placed in the automated histochemical staining instrument and the following protocol was used to stain the tissue.

TABLE 2

5	1.	Warm-up rinse buffer to 41.0° C
	2.	Rinse slide
	3.	Adjust slide volume
10	4.	Apply liquid coverslip
	5.	Warm slide chamber to 60.0° C
	6.	Rinse slide
	7.	Adjust slide volume
	8.	Apply liquid coverslip
15	9.	Rinse slide
	10.	Adjust slide volume
	11.	Apply 200µl of 0.5% potassium permanganate and incubate for 3 minutes
	12.	Apply liquid coverslip
20	13.	Rinse slide
	14.	Adjust slide volume
	15.	Apply 200µl of 0.15% oxalic acid and incubate for 3 minutes
	16.	Apply liquid coverslip
	17.	Rinse slide
25	18.	Adjust slide volume
	19.	Apply 200µl of 2.5% ferric ammonium sulfate solution and incubate for 3 minutes
	20.	Apply liquid coverslip
	21.	Rinse slide
30	22.	Apply 200µl of 0.2% silver nitrate solution and incubate for 3 minutes
	23.	Apply 200µl of ammonium hydroxide/sodium hydroxide solution and incubate for 3 minutes
	24.	Apply liquid coverslip
35	25.	Rinse slide
	26.	Rinse slide
	27.	Adjust slide volume
	28.	Apply 200µl of 10% formalin solution and incubate for 3 minutes
	29.	Apply liquid coverslip
40	30.	Rinse slide
	31.	Adjust slide volume
	32.	Apply 200µl of 0.2% gold chloride and incubate for 3 minutes
	33.	Apply liquid coverslip

34. Rinse slide
35. Adjust slide volume
36. Apply 200 $\mu$ l of 2.0% sodium thiosulfate and incubate for 3 minutes
37. Apply liquid coverslip
- 5 38. Rinse slide
39. Adjust slide volume
40. Apply 200 $\mu$ l of 1.5 g/L nuclear fast red and incubate for 3 minutes
41. Apply liquid coverslip
42. Rinse slide

10

A visual comparison between the tissue prepared as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for reticulum stain described in the AFIP Manual. Tissue stained by both techniques appeared to be identical.

15

The silver nitrate solution and ammonium hydroxide/sodium hydroxide solutions were stored at 4° C for two months, after which time the protocol in table 1 was re-run on similar tissue. A visual comparison between the tissue prepared as described above using the stored solutions was compared with manual staining of identical tissue with freshly made solutions carried out in accordance with the

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protocol for reticulum stain described in the AFIP Manual. The staining comparison demonstrated that the tissue stained by stored solutions run on the automated system were comparable or better than tissue stained manually with freshly made solutions.

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A visual comparison between the tissue prepared as described above using the stored solutions was compared with manual staining of identical tissue with one day old solutions carried out in accordance with the protocol for reticulum stain described in the AFIP manual. The staining comparison demonstrated that the tissue stained by stored solutions run on the automated system was significantly better than tissue stained manually with the one day old solutions.

**Example 3***Masson's Trichrome Stain*

5           Solutions for Masson's Trichrome stain were made as follows. Weigert's iron hematoxylin solution A was made by adding 1g of hematoxylin to 100 ml of 95% alcohol. Weigert's iron hematoxylin solution B was made by adding 4 ml of 29% aqueous ferric chloride, 95 ml of distilled water and 1 ml hydrochloric acid  
10 together. Biebrich's scarlet-acid fuchsin solution was made by combining 90 ml of 5% aqueous solution of Biebrich's scarlet with 10 ml of 10% aqueous acid fuchsin and 1 ml glacial acid. The resultant solution was mixed and filtered through a Whatman 3 filter paper.

          The 1% phosphotungstic acid solution was made by combining 1g  
15 phosphotungstic acid in 100 ml of de-ionized water. The aniline blue solution was made by adding 0.4g aniline blue to 100 ml of distilled water and 1ml of acetic acid. The acetic acid solution was made by adding 0.5ml acetic acid to 100 ml of distilled water.

**Table 3**

- 20
1. Warm-up rinse buffer to 41.0° C
  2. Rinse slide
  3. Warm slide chamber to 60.0° C
  4. Rinse slide
  - 25 5. Apply 300 µl of Weigerts B solution (1x) and incubate for 3 minutes
  6. Apply liquid coverslip
  7. Apply 200 µl of Weigerts A solution (1x) and incubate for 6 minutes
  8. Apply liquid coverslip
  9. Rinse slide
  - 30 10. Rinse slide
  11. Apply 200 µl of 5% Biebrich Scarlet solution and incubate for 9 minutes

12. Apply liquid coverslip
13. Rinse slide
14. Apply 300 $\mu$ l of 1% phosphotungstic acid solution and incubate for 6 minutes
- 5 15. Apply liquid coverslip
16. Rinse slide
17. Apply 200 $\mu$ l of 0.40% aniline blue and incubate for 3 minutes
18. Apply liquid coverslip
19. Rinse slide
- 10 20. Apply 300  $\mu$ l of 0.5% acetic acid and incubate for 3 minutes

A visual comparison between the tissue prepared as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for Masson's trichrome stain described in the AFIP Manual. The staining comparison demonstrated that the tissue stained by stored solutions when run on the automated system was comparable to tissue stained manually with freshly made solutions.

#### Example 4 *Mucicarmine Staining*

Solutions for mucicarmine staining were made as follows. Mayer's stock mucincarminine solution was made by combining 1g carmine and 0.5g anhydrous aluminum chloride in a Pyrex beaker and adding 2 ml distilled water. The solution was heated over a small flame and agitated with a glass rod for approximately 2 minutes until the solution turned purple and had the consistency of syrup. Thereafter, 100 ml of 50% ethanol was added to the syrupy mixture and the solution was incubated at room temperature for 24 h. The solution was filtered through Whatman 3 filter paper.

**TABLE 4**

1. Warm-up rinse buffer to 41.0° C
2. Rinse slide
3. Adjust slide volume
4. Apply liquid coverslip
- 5 5. Warm slide chamber to 60.0° C
6. Rinse slide
7. Adjust slide volume
8. Apply liquid coverslip
9. Start timed steps
- 10 10. Rinse slide
11. Adjust slide volume
12. Apply 300 µl of Weigerts B solution and incubate for 3 minutes
13. Apply 200 µl of Weigerts A solution and incubate for 3 minutes
14. Apply liquid coverslip
- 15 15. Rinse slide
16. Adjust slide volume
17. Apply 200 µl of mucicarmine solution and incubate for 6 minutes
18. Apply liquid coverslip
19. Rinse slide
- 20 20. Adjust slide volume
21. Apply liquid coverslip
22. Apply 100 µl of 0.1% tartrazine solution and incubate for 3 minutes
23. Apply liquid coverslip
24. Rinse slide

25 A visual comparison between the tissue prepared as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for mucicarmine stain described in the AFIP Manual. Tissue stained by both techniques appeared to be identical.

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#### **Example 5** *Gomori's Iron Stain*

A solution of 1.5g/L nuclear fast red was made by dissolving 0.15g of  
35 nuclear fast red in 5% solution of aluminum sulfate over heat. A solution of 20% hydrochloric acid was made by adding 20 ml concentrated hydrochloric acid with 80 ml distilled water. A 10% solution of potassium ferrocyanide was made by dissolving 10g potassium ferrocyanide in 100 ml distilled water.

**TABLE 5**

	1.	Warm-up rinse buffer to 41.0° C
	2.	Rinse slide
5	3.	Adjust slide volume
	4.	Apply liquid coverslip
	5.	Warm slide chamber to 60.0° C
	6.	Rinse slide
	7.	Adjust slide volume
10	8.	Apply liquid coverslip
	9.	Start timed steps
	10.	Rinse slide
	11.	Adjust slide volume
	12.	Apply 200 µl 10% potassium ferrocyanate
15	13.	Apply 200 µl of 20.0% hydrochloric acid and incubate for 9 minutes
	14.	Apply liquid coverslip
	15.	Rinse slide
	16.	Adjust slide volume
	17.	Apply 100 µl of 1.5% Nuclear fast red solution and incubate for 3 minutes
20	18.	Apply liquid coverslip
	19.	Rinse slide

A visual comparison between the tissue prepared as described above was compared with the manual staining of identical tissue carried out in accordance with the protocol for Gomori's iron stain described in the AFIP Manual. Tissue stained by both techniques appeared to be identical.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.



We claim:

1. An automated method for staining biological materials on a slide,  
comprising:
  - a) providing at least a first and second stable solution, wherein the at  
5 least first and second stable solutions when combined comprise an unstable staining  
solution;
  - b) providing a slide, wherein a biological material to be stained is  
present on the slide;
  - c) providing an automated delivery system to deliver a predetermined  
10 quantity of the at least first and second stable solutions to the biological material on  
the slide; and
  - d) sequentially applying the at least first and second stable solutions to  
the biological material on the slide using the automated delivery system.
2. The method of claim 1 further comprising mixing the at least first and second  
15 stable solutions on the biological material.
3. The method of claim 1 wherein said biological material is selected from the  
group consisting of tissue sections, tissue culture cells, cell components, including  
cell organelles, chromosomes, nucleic acids, carbohydrates, lipids, and proteins,  
smears of blood, sputum, and other body fluids, excretions and secretions, and  
20 micro-organisms including parasites, viruses, bacteria, and fungi.
4. The method of claim 1 wherein said unstable staining solution is selected  
from the group consisting of fungi staining solutions, silver staining solutions, iron  
staining solutions, iron hematoxylin solutions, trichrome staining solutions, mucin  
stains, mucicarmine staining solutions, Verhoff's staining solution, amyloid staining  
25 solutions, and Steiner staining solution.
5. The method of claim 2 wherein the mixing is accomplished by applying a  
gas stream to the at least first and second stable solutions on the biological material.
6. An automated method for silver staining biological materials on a slide,  
comprising:
  - a) providing a solution of from about 0.2% to about 1.0% silver nitrate,  
30 wherein the silver nitrate is at least first and second stable solutions when combined  
comprise an unstable staining solution;

- b) providing a solution of from about 2.0% to about 4.0% methenamine
  - c) providing a solution of from about 0.2% to about 0.6% borax
  - d) providing a slide, wherein a biological material to be stained is present on the horizontal slide;
  - 5 e) providing an automated delivery system to deliver a predetermined quantity of the silver nitrate, methenamine, and borax solutions to the biological material on the slide;
  - f) sequentially applying the silver nitrate, methenamine, and borax solutions to the biological material on the slide using the automated delivery system;
  - 10 and
  - g) mixing the silver nitrate, methenamine, and borax solutions to stain the biological material.
7. An automated method for silver staining biological materials on a slide, comprising:
- 15 a) providing a solution of from about 0.2% to about 1.0% silver nitrate;
  - b) providing a solution of from about 0.3% to about 1.0% ammonium hydroxide
  - c) providing a solution of from about 0.7% to about 1.5% sodium hydroxide
  - 20 d) providing a slide, wherein a biological material to be stained is present on the slide;
  - e) providing an automated delivery system to deliver a predetermined quantity of the silver nitrate, ammonium hydroxide, and sodium hydroxide solutions to the biological material on the slide;
  - 25 f) sequentially applying the silver nitrate, ammonium hydroxide, and sodium hydroxide solutions to the biological material on the slide using the automated delivery system; and
  - g) mixing the silver nitrate, ammonium hydroxide, and sodium hydroxide solutions to stain the biological material.
8. An automated method for trichrome or mucicarmine staining of biological materials on a slide, comprising:
- 30 a) providing a solution of from about 0.7% to about 1.5% hematoxylin;

- b) providing a solution of from about 0.5% to about 1.5% aqueous ferric chloride
- c) providing a slide, wherein a biological material to be stained is present on the slide;
- 5 d) providing an automated delivery system to deliver a predetermined quantity of the hematoxylin and aqueous ferric chloride solutions to the biological material on the slide;
- e) sequentially applying the hematoxylin and aqueous ferric chloride solutions to the biological material on the slide using the automated delivery system;
- 10 and
- f) mixing the hematoxylin and aqueous ferric chloride solutions to stain the biological material.
9. An automated method for iron staining of biological materials on a slide, comprising:
- 15 a) providing a solution of from about 8% to about 12% potassium ferrocyanate;
- b) providing a solution of from about 15% to about 30% hydrochloric acid
- c) providing a slide, wherein a biological material to be stained is present on the slide;
- 20 d) providing an automated delivery system to deliver a predetermined quantity of the potassium ferrocyanate and hydrochloric acid solutions to the biological material on the slide;
- e) sequentially applying the hematoxylin and aqueous ferric chloride solutions to the biological material on the slide using the automated delivery system;
- 25 and
- f) mixing the potassium ferrocyanate and hydrochloric acid solutions to stain the biological material.

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/12263

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/483, 35/00

US CL : 436/46, 63

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/46, 63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
U.S. PTO Scientific and Technical Information Center Library CatalogElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 3,431,886 A (MCCORMICK et al.) 03 March 1969, entire document.	1, 3, and 4
Y	US 5,650,327 A (COPELAND et al.) 22 July 1997, entire document and especially column 1, lines 10-20, and column 4, lines 35-50.	1-5
Y	MCMANNUS et al. Staining Methods Histologic and Histochemical, Paul B. Hoeber, Inc., New York, September 1960, pages 124-151, 223-245, and 361-372, especially pages 134, 138, 149, 228, 240, and 368.	3-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search  
18 AUGUST 1999Date of mailing of the international search report  
10 SEP 1999Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/12263

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG: Medline, BIOTECH, Conf. Papers, Euro, Japio  
search terms: histologic, histology, cytologic, cytology, stain, automate(d), tissue, cell, smear, silver stain, methanamine,  
borax, ammonium hydroxide, hematoxylin, eosin, potassium ferrocyanate, ferric chloride, mucicarmine, trichrome,  
verhoff, amyloid, steiner